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*Published in:*
Conservation Genetics

*DOI:*
10.1007/s10592-017-0965-3

*Publication date:*
2017

*Citation for published version (APA):*
McKendrick, L., Provan, J., Fitzpatrick, Ú., Brown, M. J. F., Murray, T. E., Stolle, E., & Paxton, R. J. (2017). Microsatellite analysis supports the existence of three cryptic species within the bumble bee Bombus lucorum sensu lato. *Conservation Genetics*, *18*(3), 573-584. https://doi.org/10.1007/s10592-017-0965-3

# Microsatellite analysis supports the existence of three cryptic species within the bumble bee *Bombus lucorum sensu lato*

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**Abstract**

Mitochondrial cytochrome oxidase I (COI) partial sequences are widely used in taxonomy for species identification. Increasingly, these sequence identities are combined with modeling approaches to delineate species. Yet the validity of species delineation based on such DNA 'barcodes' is rarely tested and may be called into question by phenomena such as ancestral polymorphisms in DNA sequences, phylogeographic divergence, mitochondrial introgression and hybridization, or distortion of mitochondrial inheritance through such factors as *Wolbachia* infection. The common and widespread European bumble bee *Bombus lucorum s. lato* contains three distinct mitochondrial DNA lineages that are assumed to represent three cryptic species, namely *Bombus cryptarum*, *B. lucorum s. str.* and *Bombus magnus*. To test whether nuclear gene pools of the three putative species were differentiated, we genotyped 304 sympatric members of the *lucorum* complex (54 *B. cryptarum* females, 168 *B. lucorum s. str.* females and 82 *B. magnus* females, as defined using mtDNA COI haplotypes) from 11 localities spread across the island of Ireland at seven nuclear microsatellite loci. Multilocus genotypes clustered into three discrete groups that largely corresponded to the three mtDNA lineages: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*. The good fit of mitochondrial haplotype to nuclear (microsatellite) genotypic data supports the view that these three bumble bee taxa are reproductively isolated species, as well as providing a vindication of species identity using so-called DNA barcodes.

**Keywords** DNA barcode; *cryptarum*; *magnus*; mitochondrial cytochrome oxidase I; STRUCTURE software; PCoA; DAPC, sympatry

## Introduction

Bumble bees (Hymenoptera: Apidae, genus *Bombus*) are of great ecological and economic importance as major pollinators of both crops and wild flowers in the Northern Hemisphere, yet they are in decline (e.g. Fitzpatrick et al. 2007; Goulson 2009; Cameron et al. 2011). Though members of the subgenus *Bombus sensu stricto* (=*Terrestribombus* Vogt) are the most abundant and widespread of all bumble bees, exhibiting a holarctic distribution (Hines 2008; Williams et al. 2008, 2012a), they can be difficult to identify in the field using the minor morphological differences that separate species (Carolan et al. 2012; Bossert 2015); the apparent abundance of members of the subgenus may mask the rarity of its morphologically indistinguishable, or cryptic, species.

In Europe, five species of *Bombus s. str*. are recognised: *B. cryptarum* (Fabricius), *B. lucorum* (L.), *B. magnus* Vogt, *B. terrestris* (L.) and *B. sporadicus* (Nylander). The taxonomic status of *B. terrestris* and *B. sporadicus* is widely accepted (Williams 1998). Difficulties arise over the other three species: *B. cryptarum, B. lucorum s. str.* and *B. magnus*, which are generally grouped to form the *lucorum* complex (*B. lucorum sensu lato*). They are cryptic species that appear very similar in colour and form, particularly as workers or males, and that are often difficult to differentiate morphologically from *B. terrestris* even as queens (Figure 1; Rasmont 1984; Rasmont et al. 1986).

Species classification based on morphological characters may not be suitable for cryptic species and genetic methods may help support species identification. Correct identification is of conservation importance because the taxonomic status of a species must be accurately established in order to assign status and direct conservation efforts (Ryder, 1986; Crandall et

al. 2000). The presence of cryptic species, however, has potentially detrimental implications since reproductively isolated groups should be managed independently of each other (Riddle et al. 2000; Palsbøll et al. 2007). To facilitate identification of cryptic species, molecular methods such as DNA barcoding can be used as a means of designating species on the basis of sequence similarity (Hebert et al. 2003). Such approaches have confirmed the view that cryptic species are particularly common in insects (e.g. Berkov 2002; Hebert et al. 2004).

In pre-DNA based studies, allozyme polymorphisms and variation in male cephalic odour bouquet supported the view that the *lucorum* complex of bumble bees comprise two or three species (reviewed in Bossert 2015). Bertsch et al. (2005) subsequently used mitochondrial cytochrome oxidase I (COI) gene sequences of specimens morphologically well-characterised as queens to show that the *lucorum* complex of bumble bees contained three distinct mitochondrial DNA (mtDNA) lineages in Europe, albeit sampling of two putative species was limited to two specimens apiece at each of two sites in Europe. Using a far larger number of samples from across Europe, including >300 from the island of Ireland, Murray et al. (2008) showed that the three mtDNA lineages exhibited considerable interspecific DNA sequence divergence (≥ 2.3%) at COI compared to intra-taxon sequence variability (≤ 1.3%), with overwhelming support for each lineage, supporting the idea that the three mtDNA lineages represent species: *B. cryptarum*, *B. lucorum s. str*. and *B. magnus*. Williams et al. (2012a) gave the distribution of these three taxa in Europe, Asia and, for *B. cryptarum*, even into North America based on COI sequences, again demonstrating overwhelming statistical support for each of the three COI lineages representing the *lucorum* complex. Murray et al. (2008) also developed a relatively quick and economic restriction enzyme based mtDNA COI marker system based on restriction fragment length polymorphisms (RFLPs) that differentiated among lineages.

Notwithstanding the success of DNA barcoding in separating species and even entire regional bee faunas (Sheffield et al. 2009; Magnacca and Brown 2010a, 2012; Schmidt et al. 2015), mitochondrial lineages may not represent independent, reproductively isolated species. Reasons include the retention of ancestral mitochondrial sequence polymorphisms, mitochondrial introgression and biased inheritance of maternal genetic markers by such factors as *Wolbachia* infection, known to be widespread in bees (Gerth et al. 2011, 2013, 2014; Gerth and Bleidorn 2013; but see Stahlhut et al. 2012), heteroplasmy (e.g., Magnacca and Brown 2010a), and associated tissue segregation of haplotypes (Magnacca and Brown 2010b). Moreover, the use of DNA barcoding or any other mitochondrial DNA marker system does not permit the detection of hybrids between taxa. This is all the more relevant for the *lucorum* complex of bumble bees, in which Carolan et al. (2012) found apparent mis-match between widely employed species-characteristic, discriminatory morphological traits of queens and mtDNA lineage among Irish specimens.

A resolution to this problem is to incorporate multilocus sequence typing into DNA barcoding studies (Gerth and Bleidorn 2013; Bossert 2015), an approach we report here for the *lucorum* complex. We used microsatellites, biparentally inherited nuclear markers, to determine the extent to which the nuclear gene pools of the *lucorum* complex taxa concur with the three mtDNA lineages of Bertsch et al. (2005) and Murray et al. (2008), with the aim of reducing taxonomic uncertainty in this group.

## Materials and Methods

### Sample collection and DNA extraction

Females (queens and workers) of *B. lucorum s.l.* were collected in 2005 and 2006 from 11 localities spread across the island of Ireland from both rural and urban environments while foraging on flowers (Table 1, Figure 2); they are a subset of the same Irish dataset originally presented in Murray et al. (2008). Individuals were either frozen or stored in 99% ethanol at 4°C prior to DNA extraction from a single leg using 10% Chelex (Walsh et al. 1991) or from half a thorax using a standard high salt protocol (Paxton et al. 1996).

Microsatellite genotyping and species identification by mitochondrial haplotyping

Individuals were genotyped at seven nuclear microsatellite loci (Supplementary Table S1) described in Stolle et al. (2011) and developed for *B. terrestris*. Forward primers included a 19 bp M13 5' tail (CACGACGTTGTAAAACGAC) and reverse primers included a 7 bp 5' tail (GTGTCTT). PCRs were carried out in a total volume of 10 µL containing 1-10 ng genomic DNA, 1 µM of 6-FAM-, TET- or HEX-labelled M13 primer (see Supplementary Table S1), 0.1 µM tailed forward primer, 1 µM reverse primer, 1x PCR reaction buffer (Promega), 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.25 U GoTaq Flexi DNA polymerase (Promega). PCRs were carried out on a MWG Primus thermal cycler using the following parameters: initial denaturation at 96 °C for 3 min followed by 35 cycles of denaturation at 96 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 3 min. Genotyping was carried out on an AB3730xl capillary genotyping system (Life Technologies; Carlsbad, California, USA). Allele sizes were scored using 500 LIZ size standards and were checked by comparison with previously sized samples.

Microsatellite genotypes were obtained at 5-7 loci for 304 females (54 *B. cryptarum*, 168 *B. lucorum s. str.* and 82 *B. magnus*; Table 1). All had already been classified to mt haplotype by RFLP analysis of a mitochondrial partial COI gene sequence by Murray et al. (2008), the results of which we use (and update by Sanger sequencing of the COI 'barcode' of eight samples) here.

Data analysis

GENEPOP (version 3.4; Raymond and Rousset 1995) was used to test for linkage disequilibrium between nuclear microsatellite loci and for deviation from Hardy-Weinberg equilibrium (HWE) at these loci. We also tested for the presence of null alleles using MICRO-CHECKER (Van Oosterhout et al. 2004). Genetic differentiation within each putative species at microsatellite loci was calculated in MICROSATELLITE ANALYSER (MSA, version 4.05 for OSX) (Dieringer and Schlötterer 1997) and isolation by distance tested using the online web service IBDWS version 3.23 (Jensen et al. 2005).

We used three approaches to determine the fit between mtDNA lineage and multilocus nuclear genotype. In the first approach, genetic clustering of individuals was assessed using a Bayesian procedure implemented in the STRUCTURE software package (version 2.3.3; Pritchard et al. 2000). The program was run without priors, and with or without the admixture ancestry model. Twenty independent runs were carried out for each model and value of *K*, the number of genetic clusters, from $K = 1$ to $K = 3$. Our rationale was to test the hypothesis of $K = 3$ clusters (representing the three species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*) versus a null hypothesis of $K = 1$ or $K = 2$ clusters (species). Each Markov chain Monte Carlo analysis used a burn-in of 50,000 followed by a further 500,000 iterations. STRUCTURE's *Q*

149 value, a probability of group membership, was calculated for each individual at $K = 3$ using
150 the admixture ancestry model.
151
152 Because our dataset suggested deviation from HWE (see results) whereas HWE is an
153 assumption of STRUCTURE, we employed two distance-based methods to test for the
154 association between genotypes and mitochondrial haplotypes, methods that do not make
155 assumptions about mating structure and that do not make *a priori* assumptions about group
156 membership. In the second approach, we visualised relationships among multilocus
157 microsatellite genotypes of the 304 females of the *lucorum* complex using principle
158 coordinate analysis (PCoA) in GENALEX version 6.5 (Peakall and Smouse 2006). In the third
159 approach, we used Discriminant Analysis of Principal Components (DAPC; Jombart et al.
160 2010) to cluster genotypes independently of *a priori* haplotype designation using the R
161 package *adegenet* version 1.4.2 (Jombart 2008) in R version 3.1.0 (R Core Team 2014). For
162 DAPC, we examined results after extracting 5, 10, 20 or 40 principal components from the
163 genotype data.
164
165 DNA sequencing to improve mt RFLP-based haplotyping
166
167 STRUCTURE *Q* values suggested that three individuals were in a different genotypic cluster to
168 that of the other individuals with the same mt RFLP haplotype (Supplementary Table S2).
169 Preliminary visualisation of the PCoA suggested that two of these three individuals and five
170 additional individuals were in a different genotypic cluster to those with the same mt RFLP
171 haplotype (Supplementary Table S2). All eight aberrant individuals were sequenced at the
172 COI 'barcode' (Hebert et al. 2003) and identified by a web-based BLASTn search against the
173 entire NCBI nucleotide database.

The original (in Murray et al. 2008) mitochondrial RFLP classification for four of these eight individuals was incorrect; two individuals with *lucorum* RFLP patterns had *cryptarum* COI DNA sequences and two individuals with *cryptarum* RFLP patterns had *magnus* COI DNA sequences (Supplementary Table S2). Though error rates in defining an individual's mt lineage using RFLPs were likely low, they nevertheless call into question the value of using RFLPs to define unambiguously the mt haplotype, as has been proposed for the *lucorum* complex of bumble bees (Murray et al. 2008; Versterlund et al. 2014). We suggest that DNA sequencing of the COI barcode is a more reliable method of defining the mt lineage in the *lucorum* complex of bumble bees in Europe and likely in other taxa, too. We recommend Sanger sequencing rather than RFLP-based inference of haplotypes in future studies of the *lucorum* complex.

The final, updated dataset is presented in Table 1 and in the Results section below.

**Results**

Approximately 10% of samples were duplicated per 96-well plate for PCR; duplicates gave identical microsatellite genotypes, suggesting very low rates of error in amplifying and calling genotypes. No consistent linkage disequilibrium (i.e. involving the same loci) was detected between any of the seven nuclear microsatellites analysed across the three putative species of the *lucorum* complex (Supplementary Table S3).

When individuals from all 11 populations were lumped together into their three putative species, *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*, loci 255 and 278 exhibited deviation

from Hardy-Weinberg equilibrium in all three putative species and loci 198, 331 and 554 deviated in two putative species (Supplementary Table S4). In most of these cases, there was evidence from MICRO-CHECKER for null alleles as the cause of the deviation (Supplementary Table S4).

Lumping individuals from different populations into a single group could lead to deviation from HWE and evidence for null alleles due to the Wahlund effect. To explore this possibility, we tested for deviation from HWE using GENEPOP and for evidence of null alleles using MICRO-CHECKER by testing each locus in each putative species at each sampling location separately (Supplementary Tables S4.1-S4.11). In the majority of cases (147 of 172 locus by species by locality combinations), genotypes did not deviate from HWE and there was no evidence of null alleles. These results suggest that all three putative species are regular outbreeders and that deviation from HWE was a consequence of having lumped individuals from different populations.

Interestingly, when we analysed deviation from HWE and sought evidence for null alleles by lumping individuals from different putative species into a single taxon, *B. lucorum s. lato*, either across all sampling localities (Supplementary Table S4) or for each sampling locality separately (Supplementary Tables S4.1-S4.11), loci were often out of HWE and showed evidence of null alleles (57 of 83 locus by location comparisons). These results provide a hint that the three putative species are differentiated in sympatry.

We tested for population genetic differentiation for each putative species separately across sampling localities for sampling site with $n \geq 5$ individuals. For each putative species, differentiation across Ireland (Figure 2) was subtle, not significantly different from zero for

*B. cryptarum* (6 locations, global $F_{ST}$ = 0.015, $P$ = 0.109) but significant for *B. lucorum s. str.* (9 locations, global $F_{ST}$ = 0.008, $P$ = 0.036) and *B. magnus* (8 locations, global $F_{ST}$ = 0.018, $P$ = 0.023). For each putative species, Isolation by Distance was not significant (statistics and population pairwise $F_{ST}$, Supplementary Table S5), probably due to low statistical power (lack of sampling sites).

Differentiation between the three putative species was high (global $F_{ST}$ = 0.268, $P$ < 0.001); all three putative species pairs were significantly differentiated (*cryptarum* versus *lucorum s. str.*: $F_{ST}$ > 0.205, $P$ < 0.001; *cryptarum* versus *magnus*: $F_{ST}$ > 0.219; $P$ < 0.001; *cryptarum* *lucorum s. str.* versus magnus: $F_{ST}$ > 0.322, $P$ < 0.001). There was no suggestion in the $F_{ST}$ values that *B. cryptarum* was closer to *B. magnus* than to *B. lucorum s. str.*, though *B. lucorum s. str.* was most distant to *B. magnus*. When we lumped the three putative species into one taxon, *B. lucorum s. lato*, differentiation across our 11 sampling sites in Ireland was insignificant ($F_{ST}$ = 0.046, n.s.). These results suggest that the three putative species are genetically well differentiated.

Differences in allele frequencies of *B. lucorum s. str.* to the other two taxa were particularly evident at locus 327 (Figure 3). Allele frequencies also differed markedly in *B. magnus* compared to the other two putative species at locus 331 (Figure 3). Yet not one allele at any of the loci was both private (restricted to a putative species) and at a sufficiently high frequency within that species to allow it to be used to discriminate readily between species.

STRUCTURE analysis at $K$ = 2 (mean likelihood Ln Pr (X|K) = -5668.6; admixture ancestry model) revealed the mitochondrial lineage corresponding to *B. lucorum s. str.* to be well differentiated from *B. cryptarum* and *B. magnus* (Figure 4), which may reflect the closer

phylogenetic affinity of the latter pair of species than either of them to *B. lucorum s. str.* (Murray et al. 2008). The nuclear gene pools of the three putative species were clearly separated at $K = 3$ (mean likelihood Ln Pr (X|K) = -5232.6**;** Figure 4), with greater model support than for $K = 1$ (mean likelihood Ln Pr (X|K) = -6880.2) or $K = 2$. Results were qualitatively the same using STRUCTURE's non-admixture model (Supplementary Figure S1).

Multilocus microsatellite genotypes of one out of the 304 individuals did not concur with the COI mtDNA species delineation; an individual with a *magnus* mitochondrial haplotype was assigned to the *cryptarum* nuclear gene pool cluster (individual Ff53, Supplementary Table S6). Two additional individuals exhibited a major split in their nuclear gene pool assignments between two putative species (individuals Ff19 and Ff27: $Q$ value $\leq 0.5$; Supplementary Tables S6 and S7). STRUCTURE assignment of the other 301 individuals to their correct mitochondrial lineage was generally with high posterior probability ($Q$ value >0.93); only 21 of the other 301 individuals (~8%) analysed exhibited a major assignment ($Q$) value of < 0.9. These results lend weight to the hypothesis that the *lucorum* complex comprises three species, with good fit of multilocus nuclear genotypes to mitochondrial haplotypes and few exceptions.

Because STRUCTURE makes the strong assumption that genotypic data are in HWE, we repeated analyses by removing three loci that suggested marked deviation from HWE: loci 255, 331 and 198 (Supplementary Table S4). Results from STRUCTURE analyses with only four loci gave largely similar results to those with the entire dataset (Supplementary Figure S2).

Genotypes of the three mtDNA lineages each formed a separate cluster when mapped in multivariate space by PCoA, with only slight overlap at the edges of clusters (Figure 5). Seven of 304 individuals did not concur with COI mtDNA species delineation. These included the three individuals (Ff19, Ff27, Ff53) whose STRUCTURE assignment suggested their genotypes did not fit with other members of the same mitochondrial lineage (Supplementary Table S7).

Clustering genotypes by DAPC also revealed three discrete clusters that largely concurred with mitochondrial lineages (20 PCs extracted from the genotype data, Figure 6), providing additional support for the hypothesis that *B. cryptarum*, *B. lucorum s. str.* and *B. magnus* are discrete species. Five of 304 individuals were at the multivariate edge of mtDNA lineages. These also included the same three aberrant individuals (Ff19, Ff27, Ff53) highlighted by STRUCTURE and PCoA (Supplementary Table S7). The same five individuals were identified as outliers when 5, 10 or 40 PCs were extracted from the genotype data for DAPC (generating 7, 7 and 5 outlier individuals respectively).

Though STRUCTURE analysis suggested *B. cyrptarum* and *B. magnus* are genetically closer to each other than either is to *B. lucorum s. str*. (Figure 4), PCoA and DAPC analyses did not support this view. Using the multivariate distance-based approaches, all three putative taxa were similarly differentiated (Figures 5 and 6).

## Discussion

Our multilocus nuclear (microsatellite) data of the *lucorum* complex of bumble bees collected in Ireland were grouped into three discrete multivariate clusters that corresponded well to the

three mitochondrial lineages formerly assigned to the putative species *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*. Our data lend weight to the hypothesis, based on mtDNA COI partial sequences, that the *lucorum* complex comprises three morphologically cryptic but reproductively isolated species: *B. cryptarum, B. lucorum s. str.* and *B. magnus*.

Mallet (1995, 2007) has persuasively argued that, in the age of genetics and genomics, a robust species definition for sexually reproducing organisms is that, when in sympatry, they form discrete genotypic clusters. The multilocus genetic differentiation of the three *lucorum* complex mitochondrial lineages that we found in our STRUCTURE analyses at $K = 3$ and using PCoA and DAPC is consistent with reciprocal monophyly of three species in sympatry.

Carstens et al. (2013) have argued that robust species delimitation should be based upon multiple, independent analyses. Here we employed three methods to differentiate nuclear gene pools of the *lucorum* complex, all of which were consistent in their identification of three discrete groups, with few outliers. STRUCTURE in particular makes the assumption of HWE, which, in our case, was violated at several loci when individuals from multiple locations were lumped together within a putative species. That STRUCTURE nevertheless identified the same outliers as PCoA and DAPC suggests it may be robust to given degree of violation of HWE in our dataset.

Though our nuclear microsatellite marker dataset suggests reproductive isolation between the three taxa of the *lucorum* complex, eight of 304 individuals possessed a multilocus genotype that did not concur with their mitochondrial haplotype. For three of these eight individuals, all three methods (STRUCTURE, PCoA and DAPC) placed them in a cluster different to that of other members of their mitochondrial lineage. One explanation for this incongruence is that

323 genotypic clusters of different species may overlap at their edges when based on a reduced
324 number of loci; use of additional microsatellite loci or genome-wide markers may resolve this
325 'lack of data' issue (Lozier and Zayed 2016). Secondly, a low degree of hybridization
326 between species may take place in the field. Individuals Ff19 and Ff27 could represent
327 hybrids because their probability of assignment (STRUCTURE *Q* value) was intermediate
328 between their mitochondrial lineage and that of another lineage. Artificial crosses between
329 members of the *lucorum* complex are needed to support this suggestion. Thirdly, aberrant
330 individuals might be a product of mitochondrial introgression; individual Ff53 could
331 represent such a case, with a *magnus* mitochondrial haplotype confirmed by Sanger
332 sequencing and a multilocus nuclear genotype assigned to *cryptarum*, *cryptarum/lucorum* and
333 *cryptarum* by STRUCTURE, PCoA and DAPC respectively. It may be of significance that all
334 three consistently aberrant individuals (Ff19, Ff27, Ff53) were collected at one site, Slieve
335 Gullion, as workers (Table 1). We note, though, that the three discrete multilocus genotypic
336 clusters we detected are unlikely to be maintained if hybridization or mtDNA introgression
337 were common and widespread. Thus our second and third explanations seem unlikely to
338 account for the mis-assigned individuals. Alternatively, if they do occur, they may not lead to
339 fertile sexual descendants (queens and males).
340
341 If, as seems likely, our first explanation is correct, it suggests that considerable effort will be
342 required for microsatellites to be used to separate among cryptic species or to detect
343 hybridization. Within European bees, there are many putative cryptic species pairs or cryptic
344 species complexes that share COI barcodes (Schmidt et al. 2015). Interspecific DNA
345 sequence divergence at COI of the *lucorum* complex in Ireland is >2.3% (Murray et al. 2008),
346 yet the 7 microsatellite loci we used to analyse 54-168 individuals per taxon were insufficient
347 to resolve unambiguously all 304 individuals. For comparison, in the cryptic Neotropical

orchid bee sister species *Euglossa dilemma* and *Euglossa viridissima*, species-typical alleles at one locus have been found to differentiate between taxa (Eltz et al. 2011), though not unambiguously. Indeed, as the number of analysed individuals per taxon increases, so too is the likelihood of detecting greater allelic diversity, reducing the probability of finding private, species-diagnostic alleles at highly variable loci.

Lack of resolution in separating between reproductively isolated nuclear gene pools using microsatellites might be due to shared ancestral polymorphisms and homoplasy caused by high mutation rates at microsatellite loci (e.g. Schlötterer 1998). Sequence divergence of other cryptic species complexes of bee at COI is considerably less than 2.3%, suggesting a more recent common ancestor than that of the *lucorum* complex; for example, three members of the *Colletes succinctus* complex (species *hederae, halophilus* and *succinctus*) share the same COI barcode (Kuhlmann et al. 2007). For these and other closely related species, a larger sample size of sympatric individuals and, possibly more importantly, a larger number of nuclear loci may be needed to separate unambiguously among reproductively isolated nuclear gene pools (e.g. using deep sequencing via genome skimming, Cossiac et al. 2016), calling into question the feasibility of using microsatellites for multilocus sequence typing so as to differentiate readily among cryptic species.

Species-specific pheromones may play an important role in mate-recognition, presumably decreasing the incidence of interspecific mating (Paterson 1985). *Bombus* male sex pheromones contain over 50 different volatile compounds derived from the labial glands that are used to scent-mark substrates and that are thought to attract unmated conspecific queens (Bergström et al. 1981; Ayasse et al. 2001). It has been previously demonstrated that males of *B. cryptarum*, *B. lucorum s. str.* and *B. magnus* differ in their cephalic secretions to the extent

that their multivariate chemical composition has been used to support specific classification of the three taxa (Bertsch 1997a; Bertsch et al. 2004, 2005; Pamilo et al. 1997). It is plausible that mate recognition based on male labial secretions plays a significant role as a prezygotic isolating mechanism in maintaining species boundaries between members of the *lucorum* complex.

The existence of three cryptic species within the *lucorum* complex of *Bombus* has several implications for conservation and management. In terms of conservation *per se*, members of the *lucorum* complex, like *B. terrestris*, are classified as of 'least concern' in Europe (Nieto et al. 2014). Nevertheless, it is highly likely that these cryptic species have been previously overlooked, and treatment of them as such should be borne in mind when addressing conservation status, particularly in light of the on-going declines in bumble bees in Europe (Goulson et al. 2005; Fitzpatrick et al. 2007; Rasmont et al. 2015). Indeed, in Ireland, *B. lucorum s. str.* is classified as 'least concern' whereas *B. cryptarum* and *B. magnus* are more cautiously and appropriately classified as 'data deficient' (Fitzpatrick et al. 2006). The three *lucorum* complex species may exhibit ecological specialization. With regard to altitude, *B. cryptarum* is has more of an upland distribution in Ireland (Murray et al. 2008) whereas *B. magnus* is considered a lowland species in Germany (von Hagen 2003). More recent analysis of their ecological associations has suggested that *B. cryptarum* and *B. magnus* prefer cooler sites in comparison to *B. lucorum* (Walters et al. 2010; Scriven et al. 2015). Phenological differences also exist; *B. cryptarum* is an early species that precedes *B. lucorum s. str.* and *B. magnus* (Bertsch 1997b; Bertsch et al. 2004), which may also play a role in preventing hybridization.

From a management perspective, it is necessary to be able to identify taxa correctly because bumble bees are important managed crop pollinators and colonies are increasingly being transplanted long distances to provide pollination services (Goulson 2003). This has become of particular conservation concern since bumble bee translocations have been implicated in colony declines, including through pathogen spillover, and in the replacement of native *Bombus* species (Inoue et al. 2008; Meeus et al. 2011; Schmid-Hempel et al. 2014). Indeed, in Asia, confusion remains over the identification of bumble bee species imported for crop pollination (Williams et al. 2012b). Importation of non-native bumble bee species not only brings risks associated with the introduction of a competitively superior congener and of a non-native's pathogens, there is also the risk of hybridization between native and introduced species. We note that inter-specific mating and hybridization can only be detected by using codominant nuclear markers such as microsatellites or SNPs; the former have been employed to demonstrate interspecific mating and hybrid inviability in crosses between native Japanese bumble bees and imported European *B. terrestris* (Kanbe et al. 2008; Tsuchida et al. 2010).

The utility of DNA barcoding versus morphological-based taxonomy in biodiversity inventorying has been hotly debated (Packer et al. 2009; Stahlhut et al. 2012; Gerth and Bleidorn 2013). Yet DNA barcodes continue to be used for species identification and even the characterization of new *Bombus* species (Williams et al. 2016). Our data vindicate their use for species identification within the *lucorum* complex.

**Acknowledgements** We thank friends and colleagues who helped to collect bumble bees across Ireland: D. Cookson, D. Dominoni, M. Kelly and S. Roos; Andreas Bertsch for use of his photographs, comments on this manuscript and encouragement to engage with the *lucorum* complex; and Robin Moritz for laboratory and intellectual support. We also thank

two anonymous reviewers and editor-in-chief as well as Shalene Jha and Christophe Praz for many insightful comments that helped improve the manuscript.

**Funding** This work was supported by a grant from the Higher Education Authority of Ireland as part of its North-South Research Programme for Peace and Reconciliation. L McKendrick thanks DARD for their financial support (a PhD stipend) and patience.

**Conflict of Interest** The authors declare that they have no conflict of interest.

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632 **Figure legends**

633

634 **Fig. 1** Queens of the four Irish members of the *Bombus s. str.* group, namely *B. terrestris* and
635 the three members of the *lucorum* complex: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*
636 (photo credit: Andreas Bertsch 2004)

637

638 **Fig. 2** Sample sites (numbers correspond to those in Table 1) and numbers of individuals
639 genotyped at seven microsatellite loci of the *lucorum* complex of bumble bees. Species
640 designation was based on mtDNA COI RFLPs and updated by Sanger sequencing

641

642 **Fig. 3** Bubble plots of allele frequencies at seven microsatellite loci of 304 individuals of the
643 three putative species of the *lucorum* complex of bumble bees from 11 sites in Ireland;
644 species designation was based on mtDNA COI haplotypes and bubble diameters reflect allele
645 frequencies which, within a species (column), sum to one

646

647 **Fig. 4** Barplot of STRUCTURE (using the admixture ancestry model) output showing
648 percentage assignment of individuals of the three *lucorum* complex species of bumble bees
649 genotyped at seven microsatellite loci to a given putative species for $K = 2$ and $K = 3$ (species
650 designation based on mtDNA COI haplotypes)

651

652 **Fig. 5** PCoA of the multilocus microsatellite genotypes of 304 *lucorum* complex bumble bees
653 from Ireland; each of the three mtDNA lineages is coded by a different shading; the three
654 individuals (Ff19, Ff27, Ff53) with low multilocus group membership (low STRUCTURE *Q*
655 value) to others of the same mtDNA lineage are labelled and circled in grey

656

**Fig. 6** DAPC of the multilocus microsatellite genotypes of 304 *lucorum* complex bumble bees from Ireland; each of the three mtDNA lineages is coded by a different shading; the three individuals (Ff19, Ff27, Ff53) with low multilocus group membership (low STRUCTURE *Q* value and DAPC assignment to a different lineage) to others of the same mtDNA lineage are labelled and circled in grey

663 **Table 1** Sample sites in Ireland and numbers of each species (as defined by mitochondrial lineage) of 304 *Bombus lucorum sensu lato* (*B.*

664 *cryptarum*, *B. lucorum s. str.* and *B. magnus*) collected and genotyped at 5-7 microsatellite loci (see Table 1 of Murray et al. (2008) for dates

665 of specimen collection); specimens were queens except where given in parentheses as workers (w)

| No | Location | Latitude (N) | Longitude (W) | *B. cryptarum* | *B. lucorum s. str.* | *B. magnus* |
|---|---|---|---|---|---|---|
| 1 | Dublin City, Co. Dublin | 53°20’22” | 06°13’38” | 11 | 28 | 0 |
| 2 | Clara, Co. Wicklow | 52°58’07” | 06°15’59” | 2 | 13 | 17 |
| 3 | Glenasmole, Co Dublin | 53°13’54” | 06°20’52” | 3 | 12 | 8 |
| 4 | Glencree, Co. Wicklow | 53°11’41” | 06°18’22” | 9 | 4 | 7 |
| 5 | Kippure, Co. Wicklow | 53°10’47” | 06°18’18” | 6 | 3 | 11 |
| 6 | Powerscourt, Co. Wicklow | 53°09’59” | 06°14’55” | 5 | 6 | 8 |
| 7 | Killarney, Co. Kerry | 52°03’48” | 09°29’55” | 0 | 19 | 12 |
| 8 | Belfast City, Co. Antrim | 54°34’13” | 05°55’09” | 6 (w=5) | 18 (w=12) | 0 |
| 9 | Cork City, Co. Cork | 51°53’54” | 08°25’29” | 1 | 40 | 0 |
| 10 | Slieve Gullion, Co. Down | 54°06’47” | 06°24’55” | 7 (all w) | 18 (all w) | 14 (all w) |
| 11 | Benbulben, Co. Sligo | 54°18’49” | 08°23’13” | 4 (all w) | 7 (all w) | 5 (all w) |
| | | | **Total** | **54** | **168** | **82** |

**Supplementary files**

**Table S1** Microsatellite primers developed for *Bombus terrestris* by Stolle et al. (2011; see Table S1 of Stolle et al. 2011) and employed in this study to genotype the *lucorum* complex of three putative species: *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*

**Table S2.** Details of the eight individuals of the *lucorum* complex whose RFLP-based mt haplotype did not concur with nuclear (microsatellite) genotypes, as defined by STRUCTURE *Q* value (Supplementary Table S6) or visually by PCoA (Figure 5). Code: the unique identifier to the specimen; Location: site of collection; RFLP ID: haplotype designation by RFLP (from Murray et al. 2008); PCoA ID: microsatellite genetic cluster membership (see Figure 5); STRUCTURE *Q* ID: microsatellite genetic cluster membership by STRUCTURE *Q* value (see Supplementary Table S6); COI-BLAST: COI barcode DNA sequence identity by BLASTn search against entire NCBI nucleotide database; GenBank Accession Number of sequence with 100% identity to Irish sample

**Table S3** Linkage disequilibrium for seven microsatellite loci in the three putative species of the *lucorum* complex of bumble bees collected at 11 sites in Ireland; columns represent respectively the pairwise combination of loci, probability of linkage disequilibrium (P) and standard error of that P value (SE), calculated in GENPOP

**Table S4** Deviation from Hardy-Weinberg equilibrium (HWE) for seven microsatellite loci in the three putative species of the *lucorum* complex of bumble bees collected from 11 sites in Ireland, including the observed and expected heterozygosity ($H_o$ and $H_{exp}$ respectively), the inbreeding coefficient, $F_{IS}$, the probability of deviation from HWE as calculated in GENEPOP

v. 4.2 (http://genepop.curtin.edu.au), and evidence for null alleles in MICRO-CHECKER; also given are $H_o$, $H_{exp}$, $F_{IS}$, and the probability of deviation from HWE for all *B. lucorum s. l.* collected from site 1 and combined into one group**. Tables S4.1-S4.11** give the same details for sampling sites 1-11 respectively.

**Table S5** Semi-matrix of pairwise estimates of $F_{ST}$ across sampling locations (a) of *B. crypatrum* (n = 6 locations), (b) *B. lucorum s. str.* (n = 9 locations) and (c) *B. magnus* (n = 8 locations), for which the number of individuals genotyped per location ≥ 5 (location notation follows that given in Figure 1); no pairwise values are significantly different from zero ($P$ < 0.05) using MSA v. 4.05 (Dieringer and Schlötterer 2003)

**Table S6** Mitochondrial lineage and multilocus probability of group membership (STRUCTURE *Q* value; admixture model) at seven microsatellite loci in the three putative species of the *lucorum* complex of bumble bees collected at 11 sites in Ireland (see Table 1 for site names and location); the one individual with aberrant correspondence between mtDNA lineage (mt sp.) and nuclear genotype (nuc cryp, nuc luc, nuc mag for *B. cryptarum*, *B. lucorum s. str.* and *B. magnus*, respectively) is highlighted in blue; the two individuals with borderline membership to their mtDNA lineage (STRUCTURE *Q* value ≤ 0.5) are highlighted in green; the 21 individuals with non-aberrant but low probability (STRUCTURE *Q* value > 0.5 and < 0.9) of membership to their mtDNA lineage are highlighted in yellow

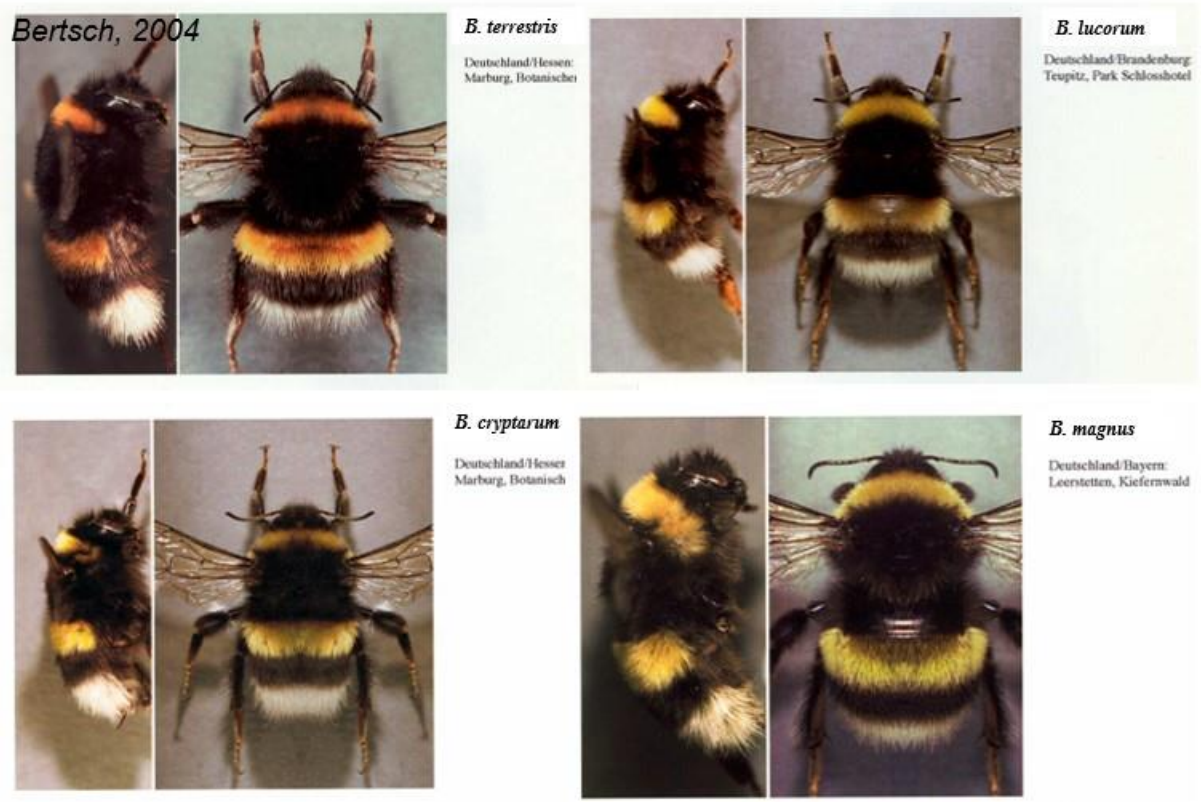
**Table S7** Details of the eight individuals of the *lucorum* complex whose updated mt haplotype did not concur with nuclear (microsatellite) genotypes, as defined by STRUCTURE *Q* value (Supplementary Table S6) or visually by PCoA (Figure 5) or by DAPC assignment (Figure 6). Code: the unique identifier to the specimen; Location: site of collection;

Haplotype ID: haplotype designation by RFLP (from Murray et al. 2008) or Sanger sequencing; STRUCTURE *Q* ID: microsatellite genetic cluster membership by STRUCTURE *Q* value (see Supplementary Table S6); PCoA ID: microsatellite genetic cluster membership (see Figure 5); DAPC ID: microsatellite genetic cluster membership (see Figure 6); comment: possible cause for lack of mt-nuclear concurrence

**Fig. S1** Barplot of STRUCTURE (using the non-admixture model) output showing percentage assignment of individuals of the three *lucorum* complex species of bumble bees genotyped at seven microsatellite loci to a given putative species for $K = 2$ and $K = 3$ (species designation based on mtDNA COI haplotypes)

**Fig. S2** Barplot of STRUCTURE (using admixture model) output showing percentage assignment of individuals of the three *lucorum* complex species of bumble bees genotyped at only four microsatellite loci to a given putative species for $K = 3$ (species designation based on mtDNA COI haplotypes). STRUCTURE criteria were the same as in the ms for all seven loci (modelling without priors, and with the admixture ancestry model, burn-in of 50,000 followed by a further 500,000 iterations). The data fit a model with K=3 better (mean likelihood Ln Pr (X|K) = -3206) than with K=2 (Ln Pr (X|K) = -3343) or K=1 (Ln Pr (X|K) = -3997). However, STRUCTURE gave two optimal results with K=3 for the reduced dataset of four loci, (a) a poorer fit (probably local) optimum in which 'cryptarum' and 'magnus' formed one group whilst 'lucorum' was split into two groups (6 of 20 runs, Ln Pr (X|K) = -3340) and (b) a better fit (probably global) optimum as in our original results presented in Fig 4 (14 of 20 runs, Ln Pr (X|K) = -3148). All 20 runs converged.

# Figure 1



# Figure 2

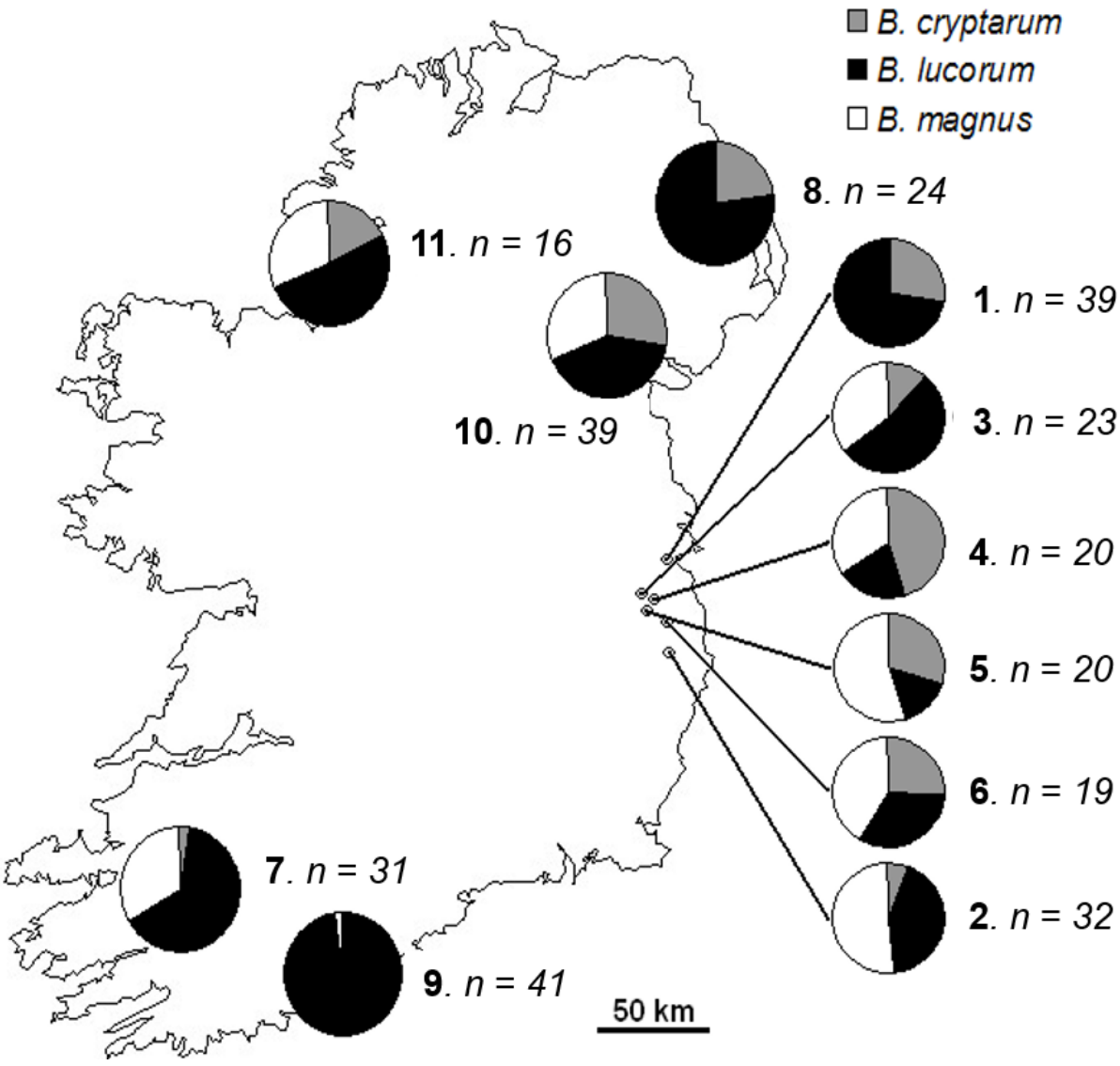


Figure 3

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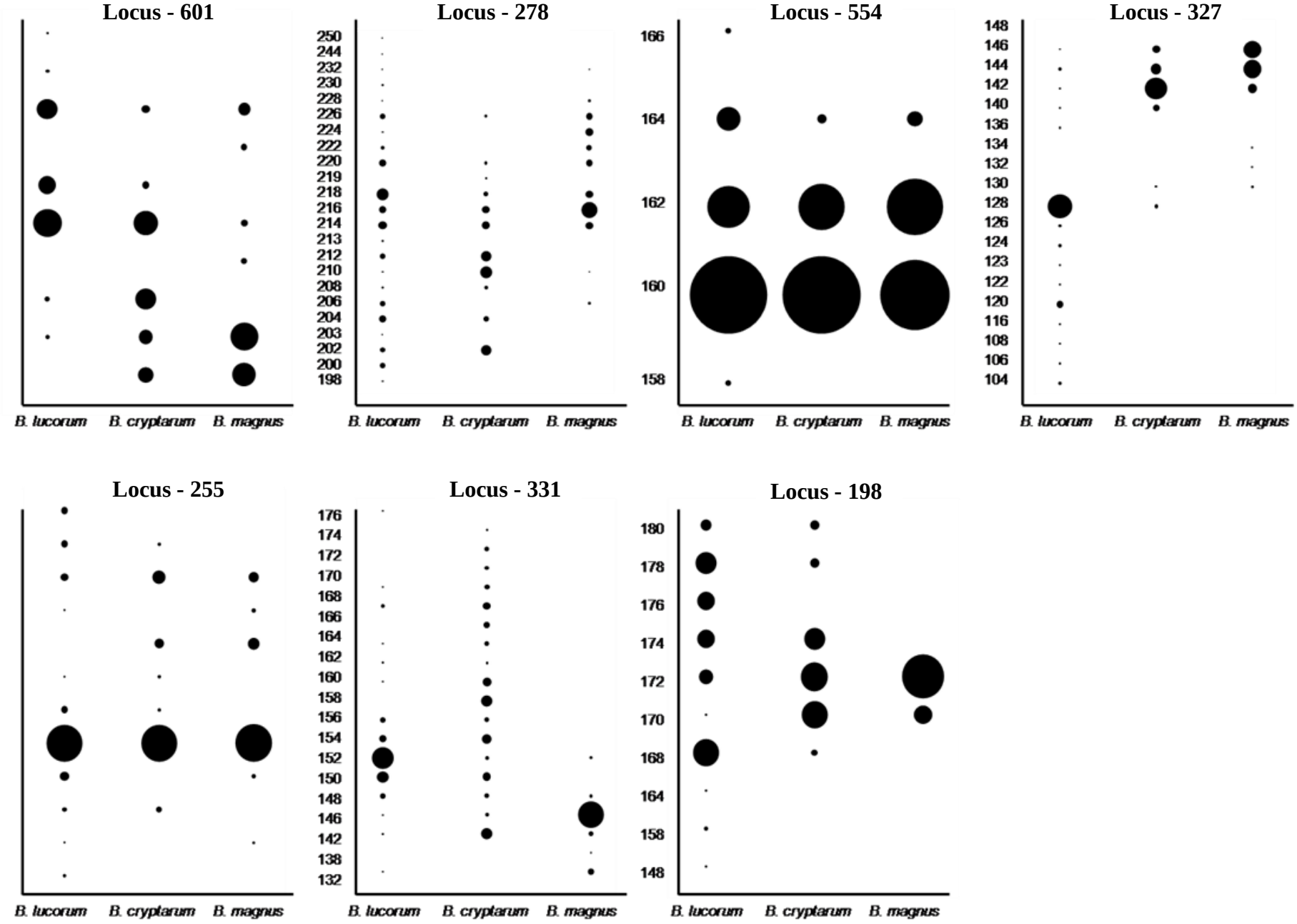
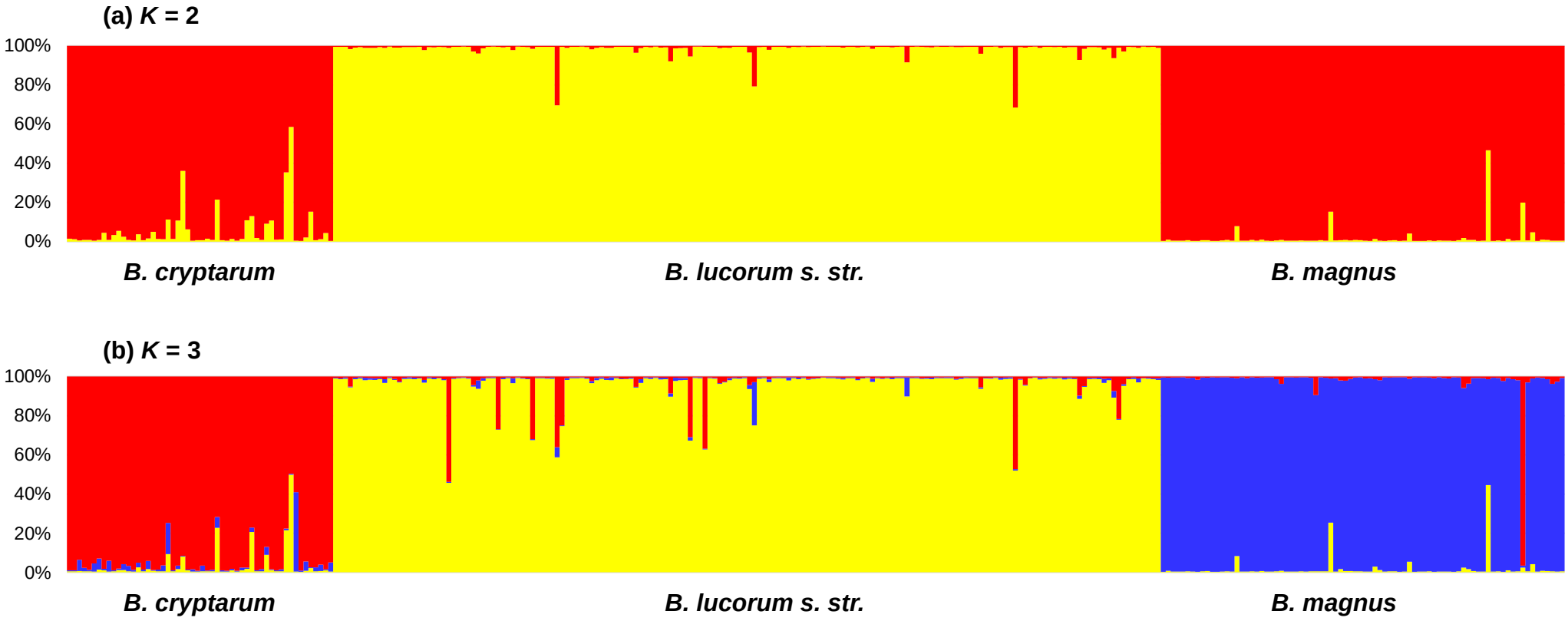
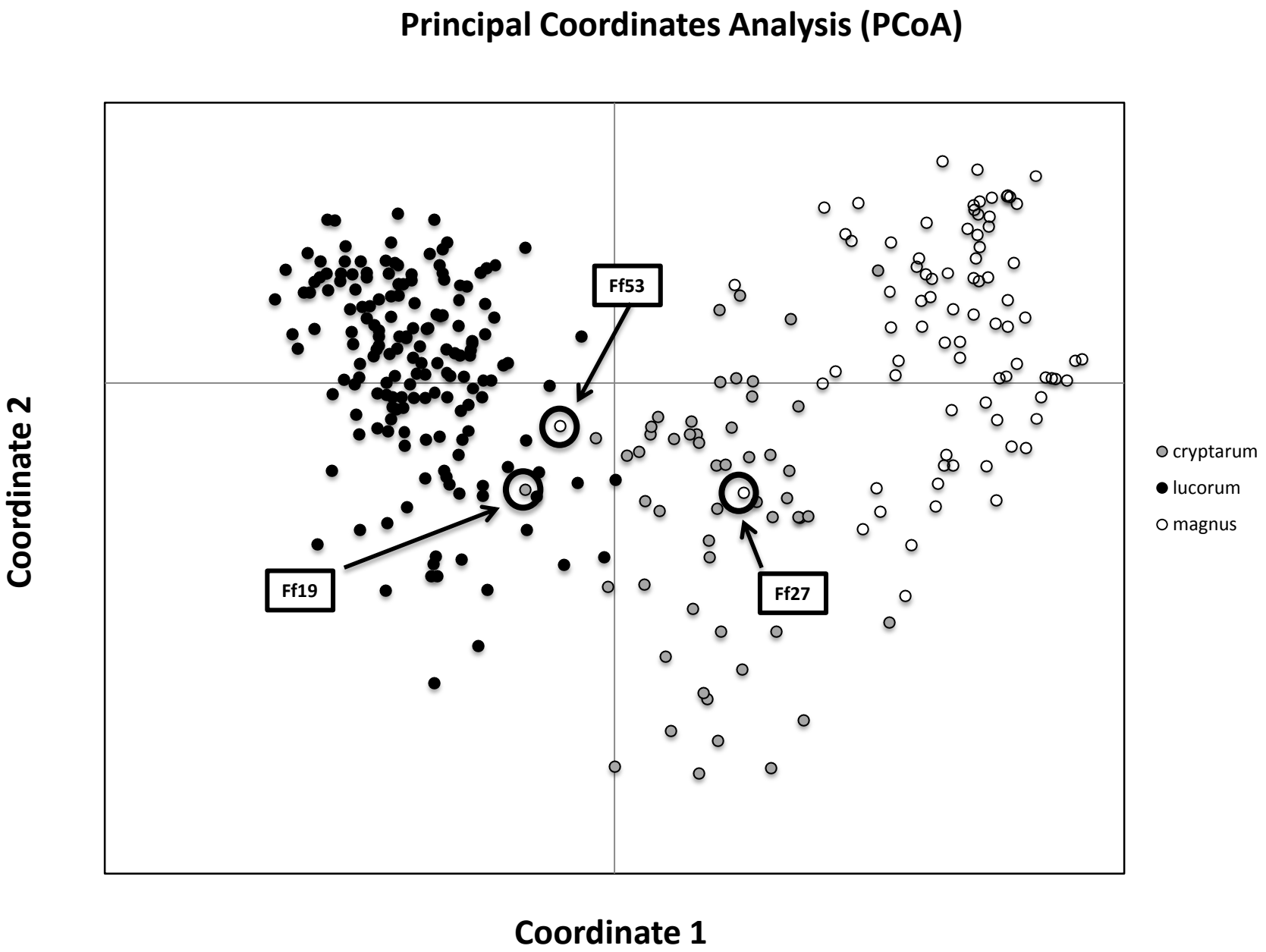
# Figure 3



# Figure 4



# Figure 5



# Figure 6